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Growth of Bacteria in Blood

Use of Cation Exchange Resins for Enhancing or Suppressing Growth *

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It has been demonstrated experimentally that small numbers of a variety of different bacteria fail to survive or multiply in normal cow or human blood or in a mixture of blood and a suitable culture medium, owing to the binding of the magnesium ion and a protein component of the antimicrobial system. However, a satisfactory and simple method has now been evolved for the rapid multiplication of Gram-negative and Gram-positive bacteria in blood without the addition of a liquid culture medium. This method involves the addition to blood of optimum amounts of hydrogen and magnesium ion exchange resins and sodium citrate.

In culturing blood for the presence of bacteria the assumption has been that any selected culture medium that grows a given bacterium well would also enhance its growth if it were present in blood added to the same medium. It has been generally presumed that the culture medium furnished the essential constituents for growth. If no growth was obtained, it was assumed that bacteria were not present.

A previous report (Huddleson, 1957) showed that multiplication of *Brucella abortus* added to mixtures of normal blood and various liquid culture media was due not to additional supplements in the culture media but to the presence of an agent or agents in the media that inactivated a component of the antimicrobial system. Rapid and abundant growth occurred in blood alone only when an exchange resin in the hydrogen form was present in a suitable amount. The pH of the blood decreased with the addition of increasing amounts of the H^+ resin. Maximum growth occurred at pH 5.9-6.2. The data indicated that rapid growth of the added bacteria was due to inactivation of one or more components of the antimicrobial system in blood by hydrogen ions.

Considerable data have been published pertaining to the bactericidal activity of whole blood or serum with reference to virulence of bacteria, and to the relationship between demonstrated activity *in vitro* and susceptibility of animals and man to various diseases. While it is known that the antimicrobial system in normal and infective blood plays an important role in preventing the growth of bacteria, there is lacking general knowledge of the changes in blood which are essential for obtaining rapid and abundant growth of bacteria therein. Furthermore, no simple method is available for inactivating a component of the system to obtain growth of widely different bacteria added to blood or those present in blood collected from infected animals and humans.

The previous study of the growth of *Br. abortus* in a mixture of blood and liquid culture media and in blood alone in the presence of one H^+ exchange resin has been expanded to include other cation exchange resins and other bacteria. The main objectives were: (*a*) to determine the role played by H^+ and other ions in altering the activity of antimicrobial factors in blood; (*b*) to determine which component of the antimicrobial system was inactivated by cationic changes in blood; and (*c*) to devise a simple and satisfactory method for growing a variety of bacteria in blood. It was hoped that the data obtained would furnish information, in addition to what is already known, as to the conditions that should prevail *in vitro* for the rapid multiplication of added bacteria and those present in blood collected from diseased animals or humans.

MATERIALS AND METHODS

The study involving blood has been confined largely to one apparently normal adult cow. There

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was no history of past *Brucella* infection or mastitis. The animal was used only for collecting blood.

Blood was collected in sterile glass containers in concentrated sodium citrate; the final concentration was 1%, and dilution of the blood due to the citrate solution was not more than 1%. Blood that was to be defibrinated was collected in sterile glass containers containing short glass rods. In the majority of the experiments, the blood, within two hours of collection, was distributed in 10-ml amounts into 50-ml sterile glass bottles containing the agent or agents to be investigated. During the study, however, it was found that blood could be used for growth studies for at least five days if it were held in a rubber-stoppered container at 3°C.

Many different commercial exchange resins in various cationic forms were studied. Hydrogen ion exchange resins of the same type and size and of similar capacity react with agents in blood in a similar manner. However, one lot of one brand of resin in the H^+ form reacted differently with blood. The properties of this resin will be discussed under experimental results.

All cation exchange resins were of the sulfonic acid type. With one exception, all were in the bead form (50-100 mesh). The exception, Duolite C-3 (H^+), was granular (10-50 mesh). It was necessary to wash the granules of Duolite C-3 thoroughly with distilled water in order to remove colloid-like particles. All were dried at 37°C for 24 hours and stored in a tightly closed bottle until ready for use. The amounts used in each experiment were based on the dry weight.

When it was necessary to change the cationic form of a resin to another cation this was accomplished by passing a solution of the salt (reagent grade) of the cation desired through the resin in an appropriate glass column. The technique employed was essentially similar to that in current use (Calmon, 1957).

The various amounts of each dry resin were weighed and placed in 50-ml serum bottles. Distilled water or a solution of a salt in the appropriate concentration was added in either 0.5-ml or 1-ml amounts to the resin for wetting and other purposes. The bottles were stoppered with cotton and sterilized at 120°C for 20 minutes.

In the employment of an H^+ exchange resin in blood it was always necessary to titrate the exchange capacity of each new lot on a resin-weight/blood-volume basis. Once the capacity of a given resin had been determined for a particular volume of blood, this operation was not repeated.

The micro-organisms used in this study were: *Brucella abortus*, *Brucella suis*, *Brucella melitensis*, *Salmonella typhosa* (Vi), *Salmonella pollorum*, *Salmonella typhimurium*, *Shigella flexneri*, *Pasteurella multocida*, *Escherichia coli* (bovine mastitis strain), *Staphylococcus aureus* (coagulase-positive strain), and *Streptococcus agalactiae* (bovine mastitis strain). All were of one colonial type, supposedly the S-type. The initial colonial type was maintained by examination of plate colonies at frequent intervals. When the slighest dissociation was detected, a fresh culture was made from a colony of the S-type. Each culture was grown on peptone M agar slants at 37°C for 24 hours. Cell suspensions were prepared in sterile distilled water containing 0.5% NaCl and 0.005% tryptose peptone and diluted in the same liquid to obtain less than 100 cells/0.2 ml (colony count).

If the agent added to blood inhibited the growth of less than 100 cells, inhibition of growth was then determined on larger numbers varying from 10^3 to 9×10^3/10 ml. By adding small or large numbers of bacteria to blood it was thought that one could measure more precisely the effects of added agents on constituents in blood which suppress or enhance growth.

After blood was added and mixed well with the other agents in the bottles, the bacteria in 0.2 ml of diluting fluid were added to each. The bottles were sealed immediately with rubber stoppers and then incubated at 37°C for 3 days. An incubation period of 3 days was determined from results obtained in previous studies. At the end of the incubation period, the contents of each bottle were thoroughly mixed, and a 1-ml aliquot of each was spread over the surface of a tryptose agar plate or diluted and plated to determine the approximate extent of multiplication of the added bacteria. This procedure, although it does not accurately portray the exact extent of multiplication, does show whether the added bacteria survived or multiplied. The dilution method was far from being an accurate one for determining the number of bacteria present as the bacterial cells often grew in clumps and were not easily dispersed into single cells.

The minus (−) sign in the tables indicates that no live bacteria were found in the 1-ml sample of blood. Otherwise the approximate number (colony count/ml) is shown.

After culturing the samples, the pH was determined by glass electrodes. This determination is important since a decrease or increase in pH due to changes in the bicarbonate-carbonate ratio or

that caused by added agents may have a marked effect on the survival, multiplication, or death of many different bacteria.

EXPERIMENTAL RESULTS

Growth of bacteria in culture medium and blood

To make the results of succeeding experiments more significant, results of two experiments are presented which demonstrate the growth and failure of growth of small numbers of different bacteria added to an excellent liquid culture medium in the presence of citrated and defibrinated cow blood.

The results in Table 1 show that while abundant growth of the bacteria employed occurred in the controls, only *Br. abortus*, *Staph. aureus*, and *Strep. agalactiae* multiplied to any extent in the presence of blood. Although *Strep. agalactiae* grew well in the presence of defibrinated blood, no living cells were recovered from the medium mixed with citrated blood. The differences in growth in the two mixtures suggested that citrate ($C_6H_5O_7^{3-}$) was involved in suppressing growth. Additional confirmation of its action was obtained in the experiments presented in Tables 2 and 3. The results in Table 2 show the effect of different concentrations of sodium citrate in a mixture of tryptose broth and defibrinated blood on the growth of four different bacteria. As the concentration was increased, its effect on the growth of Gram-negative and Gram-positive bacteria was reversed. That is, growth of the former was enhanced while that of the latter was suppressed. Similar results were obtained with five different bacteria in defibrinated blood and added $C_6H_5O_7^{3-}$ alone (Table 3).

TABLE 1

GROWTH OF BACTERIA IN CULTURE MEDIUM PLUS CITRATED OR DEFIBRINATED COW BLOOD

Bacteria	Cells added [a]	Control [b]	Citrated blood added [c]	Defibrinated blood added [c]
		Growth, 3rd day of incubation [d]		
Br. abortus	62	$> 10^9$	10^4	41
Salm. typhosa	30	$> 10^9$	—	—
Salm. pullorum	29	$> 10^9$	—	—
E. coli	21	$> 10^9$	—	—
Staph. aureus	25	$> 10^9$	$> 10^9$	$> 10^9$
Strep. agalactiae	60	$> 10^9$	—	10^4

[a] Colony count.
[b] 10 ml medium.
[c] 5 ml of blood added to 10 ml of tryptose broth in 50 ml bottles; bottles sealed during incubation.
[d] Approximate colony count per ml: —=no growth.

TABLE 2

EFFECT OF SODIUM CITRATE ON GROWTH OF BACTERIA IN A LIQUID MEDIUM IN PRESENCE OF COW BLOOD [a]

Bacteria (No. added) [b]	Blood (ml)	Sodium citrate in blood and medium (%) 0	0.2	0.6	1.2	2.4
		Growth, 3rd day [c]				
Br. abortus (35)	0	$> 10^6$	0	0	0	0
	5	—	30	$> 10^5$	$> 10^6$	$> 10^6$
Salm. typhosa (20)	0	$> 10^6$	$> 10^6$	$> 10^6$	$> 10^6$	$> 10^6$
	5	—	—	—	$> 10^5$	$> 10^5$
Strep. agalactiae (20)	0	$> 10^6$	—	—	—	—
	5	$> 10^4$	—	—	—	—
Staph. aureus (35)	0	$> 10^6$	—	—	—	—
	5	$> 10^6$	200	—	—	—

[a] 5 ml of defibrinated blood added to 20 ml of 2% tryptose in 50-ml bottles; bottles sealed during incubation.
[b] Colony count.
[c] Approximate colony count per ml; —=no growth.

These results show clearly that $C_6H_5O_7^{3-}$, even in a low concentration in blood, inhibited the growth of *Strep. agalactiae*. Higher concentrations inactivated an agent or agents of the antimicrobial system that suppressed growth of Gram-negative bacteria. If in experiments that were designed to determine the normal bactericidal or growth-inhibiting activity of citrated blood for bacteria the action of $C_6H_5O_7^{3-}$ on growth or growth-inhibiting factors was not taken into consideration, it is probable that the end results would be misinterpreted.

Sodium citrate is a chelating agent and it may combine with and alter the action of certain metal ions and proteins. If this occurred when $C_6H_5O_7^{3-}$ was added to blood, then it might be postulated that

TABLE 3
EFFECT OF SODIUM CITRATE ON GROWTH OF BACTERIA IN DEFIBRINATED BLOOD [a]

Bacteria (No. added) [b]	Sodium citrate added (%)				
	0	0.5	1	2	3
	Growth, 3rd day [c]				
Br. abortus (27)	—	—	—	2×10^3	10^3
Salm. typhosa (38)	—	—	—	2×10^3	10^4
Staph. aureus (22)	$>10^6$	$>10^6$	$>10^6$	10^3	—
Strep. agalactiae (60)	$>10^4$	—	—	—	—
Strep. agalactiae (32×10^2)	$>10^6$	2×10^3	—	—	—
E. coli (28)	—	—	—	—	10^4

[a] Sodium citrate added to 10 ml of blood in 50-ml bottles; bottles sealed during incubation.
[b] Colony count.
[c] Approximate colony count per ml.

the two Gram-positive bacteria failed to grow owing to the binding of a cation that it essential for their metabolism. For the Gram-negative ones a similar action may have occurred. In this instance $C_6H_5O_7^{3-}$ may be binding a cation, such as magnesium, considered essential for the action of the properdin-complement system (Pillemer et al., 1954). Additional evidence of the role of $C_6H_5O_7^{3-}$ and cations in suppressing or enhancing growth of bacteria added to blood are presented in the experiments that follow.

Effect of hydrogen exchange resin on growth of bacteria added to blood

The data presented in Table 4 show the degree of multiplication of four different bacteria added separately to 10-ml aliquots of both citrated and defibrinated cow blood in the presence of various quantities of an exchange resin, Duolite C-3 (H^+). The initial pH of the blood (7.6) decreased to the values shown in the table within one hour of contact. The bottles were inoculated and sealed within one hour of adding blood. Similar results were obtained in other experiments with Dowex 50-XI (H^+) and Permutit QHPF (H^+). Smaller quantities of these two resins than of Duolite C-3 were required to obtain similar pH levels and to initiate changes that enhanced multiplication of Gram-negative bacteria or inhibited growth of Gram-positive ones. This was due to the smaller size of the resin beads and to their higher exchange capacity.

Optimum changes were produced in citrated blood at pH 6.1-6.3 by the H^+ resin for the rapid growth of all Gram-negative bacteria. Evidently the H^+ level produced in blood by the H^+ resin was not the only factor that inactivated a component of the antimicrobial system in blood as one of the Gram-negative bacteria (also *Salm. pullorum* and *Sh. flexneri*) failed to survive in defibrinated blood at the same H^+ level. Although *Br. abortus* survived and grew to some extent in defibrinated blood in the presence of an H^+ resin in this experiment, in many other similar ones the added cells failed to survive.

Staph. aureus (also *Strep. agalactiae*) failed to survive in blood at pH 6.0-6.2 if $C_6H_5O_7^{3-}$ was present with the H^+ resin. In defibrinated blood, however, rapid and abundant growth of the bacteria occurred at H^+ levels from 7.6 to 6.0. Again it would appear that the change produced in blood by the H^+ resin in the presence of $C_6H_5O_7^{3-}$ which prevented the growth of Gram-positive bacteria was not due to an increase in H ions. Citrated blood (1%) of this particular cow, as well as that from six others, has never shown the least growth-inhibitory action against *Staph. aureus*. However, 10-10^3 cells of *Strep. agalactiae* always failed to survive in 10 ml of citrated cow blood. It is possible that many other varieties of *Streptococcus* do not survive in blood in the presence of sodium citrate.

The possible agents that contributed to the differences in the growth of bacteria in citrated and defibrinated blood in the presence of an H^+ resin were investigated in the experiments that follow.

Growth of bacteria in acidified cow blood

Blood is recognized as a very complex medium. In such a medium an exchange resin in the H^+ form exchanges H ions for one or more metal ions. In the exchange of H ions, one or more acids, such as HCl, H_2SO_4, H_3PO_4, and H_2CO_3 are produced. If sodium citrate is present, citric acid is produced. The amount of cations acquired by an H^+ resin suspended in a solution containing one or more salts is equi-

TABLE 4

GROWTH OF BACTERIA IN CITRATED AND DEFIBRINATED COW BLOOD IN PRESENCE OF HYDROGEN ION EXCHANGE RESIN

Blood [a]	H^+ resin added (g)	Bacteria (No. added)							
		Br. abortus (82)		*Salm. typhosa* (15)		*E. coli* (40)		*Staph. aureus* (30)	
		Growth [b]	pH	Growth [b]	pH	Growth [b]	pH	Growth [b]	pH
Citrated	0	—	7.6	—	7.6	—	7.6	$>10^7$	7.5
	0.02 [c]	—	7.5	—	7.4	—	7.5	,,	7.4
	0.05 [c]	—	7.1	—	7.2	—	7.3	,,	7.2
	0.1 [c]	10^3	6.8	—	7.0	—	7.1	,,	6.9
	0.2 [c]	10^4	6.5	—	6.7	$>10^7$	6.9	,,	6.7
	0.35 [c]	$>10^7$	6.1	$>10^7$	6.3	$>10^7$	6.7	—	6.2
Defibrinated	0	2	7.5	—	7.6	—	7.6	$>10^7$	7.5
	0.05 [d]	5	7.5	—	7.3	—	7.4	,,	7.3
	0.1 [d]	3×10^2	7.2	—	7.1	—	7.0	,,	7.1
	0.2 [d]	10^3	6.8	—	6.7	$>10^7$	6.9	,,	6.7
	0.35 [d]	$>10^6$	6.3	—	6.2	$>10^7$	6.3	,,	6.2

[a] 10 ml of blood added to each of 50-ml bottles containing amounts of Duolite C-3.
[b] Approximate colony count per ml after 3 days' incubation.
[c] 0.1 g sodium citrate in 1 ml H_2O added before autoclaving.
[d] 1 ml H_2O added before autoclaving; bacteria added 1 hour after blood.

valent to the H ions exchanged. The physical and chemical properties of the resin determine the nature of the cation sorbed in the exchange.

A series of experiments was made to determine whether the type of acid produced in blood by the presence of an H^+ exchange resin accounted for differences in the growth of bacteria in blood lowered to pH near 6.0. In each of the experiments, four inorganic and three organic acids were added separately to 10-ml aliquots of both citrated and defibrinated cow blood in sufficient concentration to obtain a pH of 5.9-6.3. The volume of each acid added was 0.2-0.3 ml. The aliquots of blood after acidifying were inoculated separately with 35-88 cells of five different bacteria. The bottles were sealed and incubated for three days.

The results obtained in one of the experiments in which citrated blood and five different bacteria were employed are set forth in Table 5. Citric acid was the only one of the seven acids added to blood that completely suppressed the activity of the antimicrobial system for all the Gram-negative bacteria. The results obtained with *Salm. pullorum*, *Salm. typhimurium*, and *Sh. flexneri* were similar to those obtained with *Salm. typhosa*. The fact that cells of only *E. coli* multiplied rapidly in citrated blood in the presence of all acids except one (HCl) would indicate that not all components of the normal antimicrobial system in blood operate in the same manner for different Gram-negative bacteria.

Citric acid, which produced sufficient changes in blood for the growth of all Gram-negative bacteria, was the only one that induced adequate changes which prevented growth of both *Staph. aureus* and *Strep. agalactiae*. Results similar to those shown in Table 5 were also obtained with defibrinated blood.

TABLE 5
GROWTH OF BACTERIA IN ACIDIFIED COW BLOOD (CITRATED) [a]

Bottle No. [b]	Acid added	Bacteria added				
		Br. abortus	*Salm. typhosa*	*E. coli*	*Staph. aureus*	*Strep. agalactiae*
		Growth, 3rd day [c]				
1	0	—	—	—	$> 10^7$	—
2	Citric	$> 10^6$	$> 10^6$	$> 10^7$	—	—
3	Lactic	—	$> 10^6$	$> 10^7$	$> 10^7$	—
4	Pyruvic	—	—	$> 10^7$	10^3	—
5	Hydrochloric	—	—	10^2	$> 10^7$	$> 2 \times 10^3$
6	Sulfuric	95	—	$> 10^7$	$> 10^7$	—
7	Nitric	$> 10^4$	—	$> 10^7$	$> 10^7$	—
8	Phosphoric	10^2	—	$> 10^7$	$> 10^7$	$> 10^5$

[a] Inoculum varied from 35 to 88 cells.
[b] Acid added to 10 ml blood in 50-ml bottles and sealed during incubation.
[c] Approximate colony count per ml. Final pH of each blood unit except control varied from 5.9 to 6.3; control was 7.6.

Since the aliquots of blood were adjusted to approximately the same pH level with the different acids, it would now appear that growth or prevention of growth of micro-organisms in blood in the presence of an H^+ exchange resin depends upon the type of acid or acids formed.

If all bactericidal activity of cow blood were due to the properdin system, it would also be logical to assume that rapid growth of Gram-negative bacteria was due to binding of magnesium ions by citric acid, as this ion is essential for the activity of the system (Pillemer et al., 1954). It is conceivable that the failure of *Staph. aureus* and *Strep. agalactiae* to grow in blood in the presence of citric acid was also due to the binding of a particular metal ion such as Mg^{2+}, which is essential for growth.

Effect of sodium, potassium, and magnesium ions on growth of bacteria in blood

To determine whether citric acid was binding a metal ion in blood essential for the activity of the antimicrobial system against Gram-negative bacteria and required for the growth of *Staph. aureus* and *Strep. agalactiae*, 10-ml aliquots of blood were exposed for two hours to a sufficient amount of an H^+ resin plus 0.1 g of sodium citrate to obtain a pH of 6.0-6.2. Each aliquot was removed from the resin and added to separate bottles containing 0.01, 0.05, 0.1, 1, and 2 g of an exchange resin in the Na^+, K^+ or Mg^{2+} form. Each aliquot was then inoculated separately with less than 50 cells of *Br. abortus*, *Salm. typhosa*, *Salm. pullorum*, *Staph. aureus*, and *Strep. agalactiae*, sealed and incubated for the usual length of time. The pH remained near 6.0.

The Gram-negative bacteria grew rapidly in all treated aliquots of blood. This finding would indicate that the loss of antimicrobial activity in blood treated with an H^+ resin and sodium citrate was not due entirely to the inactivity of Na^+, K^+ or Mg^{2+}. The only treated aliquots in which growth of the two Gram-positive bacteria occurred were those containing the resin in the Mg^{2+} form.

Effect of magnesium ions on growth of Gram-positive bacteria in blood

A sufficient amount of an H^+ exchange resin and 0.1 g of sodium citrate were added to 10-ml aliquots

of citrated cow blood to obtain a pH near 6.0. After one hour's contact the aliquots of blood were removed from the resin and transferred to separate bottles. To one lot of aliquots were added various quantities of an exchange resin in the Mg^{2+} form or various concentrations of $MgCl_2$. The other treated aliquots were adjusted to pH 7.5 with either 2 N Na_2CO_3 or 5 N NaOH. To several of the aliquots adjusted with NaOH were added various quantities of an Mg^{2+} exchange resin or various concentrations of $MgCl_2$. All treated aliquots as well as controls were inoculated with less than 100 cells of either *Staph. aureus* or *Strep. agalactiae*, sealed, and incubated at 37°C for three days.

It is apparent from the data presented in Table 6 that the failure of the two Gram-positive bacteria to grow in blood previously treated with an H^+ exchange resin and sodium citrate was due to the binding of Mg^{2+} normally present. When sufficient Mg^{2+} was again made available either from an exchange resin or from the chloride while the blood was near pH 6.0, abundant growth occurred.

By adjusting the pH of blood to 7.5 with Na_2CO_3, Mg^{2+} bound to $C_6H_5O_7^{3-}$ was released as soluble magnesium bicarbonate and became available for growth. Magnesium was still not available after adjusting the pH to 7.5 with NaOH owing to the formation of a very slightly soluble hydroxide of magnesium.

TABLE 6

EFFECT OF MAGNESIUM ION AND pH ADJUSTMENT ON GROWTH OF *STAPH. AUREUS* AND *STREP. AGALACTIAE* IN CITRATED COW BLOOD

H^+ resin added [a]	Blood removed [b]	pH to 7.5 with:	Mg^{2+} added [c]	Bacteria (No. added)	3rd day	
					Growth [d]	pH
None	No	—	—	*Staph. aureus* (40)	$> 10^7$	7.6
Duolite C-3, 0.38 g	Yes	—	—		—	6.1
		—	Resin, 0.02 g		85	6.1
		—	Resin, 0.05 g		$> 10^7$	6.2
		—	$MgCl_2$, 10^{-3} M		$> 10^6$	6.2
		Na_2CO_3	—		$> 10^7$	7.4
		NaOH	—		6	7.5
None	No	—	—	*Strep. agalactiae* (97)	—	7.6
Duolite C-3, 0.38	Yes	—	—		—	6.2
		—	Resin, 0.05 g		$> 10^6$	6.2
		—	$MgCl_2$, 10^{-3} M		—	6.2
		Na_2CO_3	—		200	7.5
		NaOH	—		—	7.5

[a] 10 ml blood added to resin and 0.1 g sodium citrate in 50-ml bottles, pH 5.9.
[b] Blood removed after 2 hours and added to bottles for pH adjustment.
[c] Mg^{2+} resin or $MgCl_2,6H_2O$ added after pH adjustment; bottles sealed during incubation.
[d] Approximate colony count per ml.

Reactivation of antimicrobial activity of blood

It has repeatedly been demonstrated that the normal antimicrobial activity of cow blood is inactivated by treatment with an H^+ exchange resin and sodium citrate. It was considered of interest to determine whether or not the original activity could be restored and to identify the nature of the agents involved.

In order to attain these two objectives, 10-ml aliquots of citrated blood were added to a sufficient amount of H^+ resin plus 0.1 g of sodium citrate to lower the pH to 5.9. After a two-hour contact period each aliquot was removed from the resin and added to separate bottles. One series of these contained various amounts of an Mg^{2+} exchange resin and another contained sufficient 2 N Na_2CO_3 or 5 N NaOH to raise the pH to 7.5. Blood in one series of aliquots adjusted to pH 7.5 was added to a third series of bottles containing either various amounts of an Mg^{2+} exchange resin or sufficient $MgCl_2$ to obtain molar concentrations of 10^{-2}, 10^{-3}, and 10^{-4}. Each was inoculated with less than 50 bacterial cells.

The results of one of the foregoing experiments in which cells of *Salm. typhosa* were used to determine possible reactivation of bactericidal activity are presented in Table 7. In other similar experiments cells of *Br. abortus*, *Salm. pullorum*, and *Sh. flexneri* were used. The results obtained paralleled those shown in Table 7. The data show clearly that the factors involved in the normal bactericidal activity of blood were not permanently inactivated by the chemical changes that occurred following the addition of an H^+ exchange resin and $C_6H_5O_7^{3-}$.

If it is assumed that the bactericidal system in normal cow blood is due to the combined action of properdin, complement, and Mg^{2+}, then any agent which suppresses the action of one component blocks the action of the entire system. The results in Table 7 indicate that Mg^{2+} was involved since activity was restored after the pH was adjusted to 7.5 with Na_2CO_3. After adding Na_2CO_3, Mg^{2+} normally present in blood was released from $C_6H_5O_7^{3-}$ as a soluble bicarbonate. However, failure to restore activity by adding sufficient Mg^{2+} while the blood was at pH 5.9 would indicate that another component

TABLE 7
REACTIVATION OF ANTIMICROBIAL ACTIVITY OF COW BLOOD FOR *SALM. TYPHOSA*

H^+ resin added [a]	Blood removed [b]	pH to 7.5 with:	Mg^{2+} added [c]	3rd day	
				Growth [d]	pH
None	No	—	—	—	7.6
Duolite C-3 0.39 g	Yes	—	—	$> 10^7$	5.9
		—	Resin, 0.1 g	$> 10^7$	5.9
		—	$MgCl_2$, 10^{-2} M	$> 10^7$	5.9
		Na_2CO_3	—	—	7.5
		NaOH	—	$> 10^7$	7.5
		NaOH	Resin, 0.1 g	—	7.5
		NaOH	$MgCl_2$, 10^{-3} M	—	7.5
		NaOH	$MgCl_2$, 10^{-4} M	$> 10^6$	7.5

[a] 10 ml blood added to resin and 0.1 g sodium citrate in 50-ml bottles.
[b] Blood removed after 2 hours and transferred to bottles for pH adjustment.
[c] Mg^{2+} resin or molar solution of $MgCl_2,6H_2O$ after pH adjustment; inoculum, 38 cells.
[d] Approximate colony count per ml.

TABLE 8
GROWTH OF BACTERIA IN CITRATED COW BLOOD IN PRESENCE OF HYDROGEN AND MAGNESIUM ION EXCHANGE RESINS

Resins added (g) [a]	Bacteria (No. added)									
	Br. abortus (29)		*Salm. typhosa* (30)		*E. coli* (28)		*Staph. aureus* (22)		*Strep. agalactiae* (93)	
	Growth [b]	pH	Growth [b]	pH	Growth [b]	pH	Growth [b]	pH	Growth [b]	pH
None	—	7.6	—	7.6	—	7.6	$> 10^7$	7.5	—	7.6
H^+, 0.38	$> 10^7$	6.0	$> 10^7$	6.1	$> 10^7$	6.2	—	6.0	—	6.1
H^+, 0.38 Mg^{2+}, 0.02	$> 10^7$	6.0	$> 10^7$	6.1	$> 10^7$	6.0	—	6.0	—	6.1
H^+, 0.38 Mg^{2+}, 0.05-0.1	$> 10^7$	6.0	$> 10^7$	6.1	$> 10^7$	6.0	$> 10^7$	6.1	$> 10^7$	5.8

[a] 10 ml of blood added to resins + 0.1 g sodium citrate in 50-ml bottles, each sealed after inoculation.
[b] Approximate colony count per ml at 3rd day of incubation.

of the bactericidal system in addition to Mg^{2+} was bound by $C_6H_5O_7^{3-}$ and as a result was inactive. It is presumed that a bound protein component essential for the activity of the bactericidal system was also released from $C_6H_5O_7^{3-}$ by Na_2CO_3. Sufficient Mg^{2+} to restore activity of the bactericidal system was not available after adding NaOH. By supplying Mg^{2+} as an exchange resin or as chloride after adding NaOH to blood, the bactericidal activity of the system was restored.

Growth of bacteria in citrated blood in presence of both H^+ and Mg^{2+} exchange resins

The results of previous experiments revealed that rapid and abundant growth of five Gram-negative bacteria could be obtained in blood treated with an H^+ exchange resin and $C_6H_5O_7^{3-}$. Two Gram-positive bacteria failed to grow under similar conditions unless a sufficient amount of Mg^{2+} was available. It was therefore considered important to determine whether or not the presence of an adequate amount of both an H^+ and Mg^{2+} resin plus $C_6H_5O_7^{3-}$ in blood would create sufficient changes in constituents therein so that rapid and abundant growth of either Gram-negative or Gram-positive bacteria would occur.

The results of one experiment of this nature are set forth in Table 8. In this experiment 10-ml aliquots of citrated blood were added to sterile bottles containing 0.38 g of an H^+ and various amounts of an Mg^{2+} exchange resin. In addition, each bottle contained 0.1 g of sodium citrate (1 ml of a 10% solution). After one hour's contact with the resins, the aliquots of blood were inoculated separately with less than 100 cells of five different bacteria.

The data show that the presence of the three agents in blood created adequate changes for the rapid and abundant growth of the five different bacteria. The chemical changes produced in blood by the H^+ resin plus $C_6H_5O_7^{3-}$ altered components of the normal antimicrobial system to such a degree that that system was no longer capable of suppressing the growth of Gram-negative bacteria. Growth of the two Gram-positive bacteria occurred when sufficient Mg^{2+} was available from the added exchange resin. The added Mg^{2+} replaced the amount normally present, which was bound by $C_6H_5O_7^{3-}$. Magnesium ions in blood at pH near 6.0 in the presence of $C_6H_5O_7^{3-}$ do not reactivate the antimicrobial system for Gram-negative bacteria. With this procedure, the other bacteria employed in this study were also found to multiply as rapidly in blood as those recorded in Table 8.

While making a comparative study of different H^+ exchange resins on the growth of bacteria in blood, only one resin, designated as Rezex 5H (H^+), was found which had properties distinctly different from the others. Small numbers of different Gram-negative bacteria added to blood treated with this resin and $C_6H_5O_7^{3-}$ failed to survive and grow. Gram-positive bacteria (*Staph. aureus*), however, grew

rapidly in blood in its presence. Samples of ten other lots of Rezex 5H were examined and found to produce changes in blood similar to those produced by the other H^+ resins. The property possessed by the one lot of Rezex 5H which accounted for its difference has not been determined.

Effect of manganese and cobalt ions on the growth of bacteria in defibrinated cow blood

It has been reported (Wardlaw et al., 1958) that either Mn^{2+} or Co^{2+} can be substituted for Mg^{2+} in the properdin-complement system in blood serum provided a slightly higher molar concentration of either is used. Manganese and cobalt ion exchange resins and various molar concentrations of the chlorides were added to blood for the purpose of determining their effect on the growth of several different bacteria rather than for the replacement of Mg^{2+}. The Mn^{2+} and Co^{2+} resins were prepared by treating high-capacity H^+ exchange resins (Dowex 50-XI, Permutit QHPF, Duolite C-3) with 0.1 M solutions of the chlorides of each metal (Calmon, 1957).

Ten-millilitre aliquots of defibrinated blood were added to various weights of each exchange resin and to 0.2 ml of various molar concentrations of the chlorides of Mn^{2+} or Co^{2+} in 50-ml bottles. After one hour's contact the bacterial cells were added.

It was surprising to find that Mn^{2+} released from a small amount of exchange resin (Dowex 50-XI) as well as that available from a 5×10^{-6} M concentration of the chloride enhanced the growth of *Brucella* (Table 9). The increase in growth of *Br. suis* and *Br. melitensis* was especially noticeable. Rapid and abundant growth of *E. coli* occurred in blood in the presence of the Mn^{2+} resin when large numbers of cells (48×10^3) were added. In many trials, 4×10^4 cells of *E. coli* failed to survive in 10 ml of blood of this cow. Similar amounts of an Mn^{2+} exchange resin and molar concentrations of $MnCl_2$ failed to change the bactericidal activity of cow blood for *Salm. typhosa*, *Salm. pullorum*, and *Sh. flexneri*. Manganese ions released from 0.02 g of resin in blood inhibited the growth of *Staph. aureus*, but had no apparent inhibiting effect on the growth of *Strep. agalactiae*.

The nature of the change produced in blood from added Mn^{2+} that enhanced the growth of *Brucella*

TABLE 9

GROWTH OF BACTERIA IN DEFIBRINATED COW BLOOD IN PRESENCE OF MAGNESIUM EXCHANGE RESIN OR $MnCl_2$

Bacteria (No. added) [b]	Mn^{2+} resin added (g)						Molar $MnCl_2$ [c]				
	0	0.005	0.01	0.02	0.05	0.1	5×10^{-6}	10^{-4}	10^{-3}	5×10^{-3}	10^{-2}
	Growth, 3rd day [d]										
Br. abortus (50)	2	29	10^2	10^3	10^4	10^4	32	55	$>10^6$	$>10^6$	52
Br. suis (50)	28	3×10^2	2×10^3	$>10^5$	$>10^5$		3×10^3	$>10^6$	$>10^6$	$>10^6$	15
Br. melitensis (70)	60	10^2	5×10^2	2×10^3	3×10^2		50	10^3	$>10^6$	250	36
Staph. aureus (2×10^3)	$>10^7$		10^5	66	6	—	—	$>10^7$	$>10^7$	$>10^6$	75
E. coli (19)	0		$>10^7$	—	—	—	—	—	—	—	—
E. coli (48×10^3)	—		$>10^7$	$>10^7$	$>10^7$	$>10^7$	—	—	—	—	$>10^7$

[a] 10 ml blood added to 50-ml bottles containing resin of $MnCl_2,4H_2O$.
[b] 1 hour after adding blood.
[c] Molar concentration in blood; bottles sealed during incubation.
[d] Approximately colony count per ml.

TABLE 10
GROWTH OF BACTERIA IN DEFIBRINATED COW BLOOD IN PRESENCE OF COBALT EXCHANGE RESIN OR $CoCl_2$ [a]

Bacteria (No. added)	Co^{2+} resin added (g)					Molar $CoCl_2$ added [b]		
	0	0.01	0.02	0.05	0.1	10^{-5}	10^{-4}	10^{-3}
	Growth, 3rd day [c]							
Br. abortus (6×10^3)	2×10^3	2	—	—	—	$>10^6$	10^4	—
Staph. aureus (4×10^3)	$>10^7$		—	—	—	$>10^7$	$>10^7$	80
Strep. agalactiae (9×10^3)	$>10^7$	4	—	—	—	$>10^6$	$>10^4$	5×10^2

[a] 10 ml blood added to 50-ml bottles containing resin or $CoCl_2,6H_2O$ one hour before inoculation.
[b] Molar concentration in blood; bottles sealed during incubation.
[c] Approximate colony count per ml.

and *E. coli* has not yet been determined. According to studies pertaining to the nutritional requirements of *Brucella* in culture media, Mn^{2+} is not essential (Gerhardt, 1958).

The results in Table 10 illustrate the effect of Co^{2+} on the growth of large numbers of *Br. abortus*, *Staph. aureus* and *Strep. agalactiae* in defibrinated blood. Various amounts of a Co^{2+} exchange resin (Dowex 50-XI) and various molar concentrations of $CoCl_2$ were added to 10-ml aliquots of defibrinated cow blood. After one hour's contact each aliquot was inoculated separately with large numbers of *Br. abortus*, *Staph. aureus*, or *Strep. agalactiae*. The results show that Co^{2+} itself is highly bactericidal or enhances the bactericidal activity of components in blood. Growth-inhibiting action of Co^{2+} available in the chloride form was less than that available from the exchange resin. The mechanism of the growth-inhibiting action of Co^{2+} on bacteria added to blood is still to be determined.

DISCUSSION

The data in the first part of this study show that the growth of small numbers of many different Gram-negative bacteria in freshly collected blood was not always enhanced by a liquid culture medium. It also points up the importance of recognizing and considering the role played by antimicrobial agents in blood in preventing growth of added bacteria or those present in blood from an infected patient.

Observations and reports generally have indicated that blood from certain human patients infected with *Brucella* is devoid of bactericidal activity for this group of bacteria. Hall (1950), however, has demonstrated that blood or serum of *Brucella*-infected patients shows considerable activity against added *Brucella* cells during the first three months of infection. After the third month activity was no longer demonstrated. He attributed the loss of activity to the late appearance of an antimicrobial blocking factor in blood.

Over the two years during which the blood of one animal was studied the most important and uncontrollable variable encountered was the variation in the antimicrobial activity of both citrated and defibrinated blood for *Brucella*. Growth of *Br. abortus* (inocula of 80 cells or less) was not inhibited in 10 ml of blood from approximately 10% of the 200 different samplings, while the growth of *Br. suis* and *Br. melitensis* was inhibited in only 10% of the samplings. Others have noted that the antimicrobial activity of normal cow blood for *Brucella* varies considerably (Mackie et al., 1932; Irwin et al., 1936).

No attempt was made to distinguish natural antimicrobial agents of a protein nature in blood from those acquired from previous exposures to various bacteria. One could hardly surmise that the latter were not present for certain bacteria since members of the bovine species are intermittently exposed to many different Gram-negative and Gram-positive bacteria throughout their life. Furthermore, no

attempt was made to distinguish variations in antimicrobial activity due to agents of a protein nature or to metal ions.

Following the addition of an H^+ exchange resin of high capacity to blood, there is a loss of metal ions present and an increase in acidity from the interchange of H^+. The kind and amount of metal ion attracted to the resin depends upon the nature of the resin, the quantity of resin added, and the salts present in blood at the time. The nature of the acid produced depends upon the form in which the metal ions are present at the time exchange takes place.

It is known that one or more of several acids may be formed in blood in the interchange of H^+ on a cation exchange resin with cations of salts normally present or added $C_6H_5O_7^{3-}$. The results obtained in blood acidified with different acids and inoculated with different bacteria indicated that growth or absence of growth depended on the type of acid added and the nature of the reaction with blood constituents. Such reactions may account for the differences between the growth of bacteria in defibrinated blood in the presence of an H^+ resin and that which occurred if $C_6H_5O_7^{3-}$ was also present.

It appears that the H^+ exchange resin plays only a secondary but an important role in bringing about chemical changes in blood which inhibit the growth of Gram-positive bacteria. Citrate must also be present at the same time to bind Mg^{2+} normally present. When combined with $C_6H_5O_7^{3-}$ or OH^-, Mg^{2+} is not in an available form. This cation appears to be essential for the growth of small numbers of *Staph. aureus* and *Strep. agalactiae* in blood. Reactivation of growth of *Staph. aureus* in blood at pH 6.0 required the addition of approximately 24 μg/ml of Mg^{2+}.

Sufficient $C_6H_5O_7^{3-}$ in blood in the form of a sodium salt or acid suppresses the activity of the normal bactericidal system for Gram-negative bacteria. Apparently the activity of at least two components of the system is altered. One of these is Mg^{2+} and the other may be complement. Properdin, the third component of the system, is probably not involved as its activity is unaltered above pH 5.2 (Pillemer, 1956).

It is postulated that both agents were bound to $C_6H_5O_7^{3-}$ at pH near 6.0 and in this form were inactive. By adjusting the pH of blood above 7.0 with Na_2CO_3, both were released from citrate and thus reactivated the bactericidal system.

After adding both an H^+ and Mg^{2+} exchange resin to blood, blood alone became an excellent culture medium for the rapid growth of both Gram-negative and Gram-positive bacteria even when only a small number were present. Growth depended on the presence of optimum amounts of the exchange resins and $C_6H_5O_7^{3-}$. Those bacteria requiring more than atmospheric carbon dioxide for growth also multiplied rapidly if the containers were kept closed with rubber stoppers during the incubation period. Additional carbon dioxide is obtained by the decomposition of sodium bicarbonate in blood.

It was shown in this study that only 0.18 μg/ml of Mn^{2+} in the chloride form was required to enhance growth of *Brucella* in defibrinated blood. This amount of Mn^{2+} is within the range of that normally present in blood of cattle (Blakemore et al., 1937; Bentley & Phillips, 1951).

An increase or decrease of Mn^{2+} in normal blood from the intake of food in which this ion is high or low could affect the results of bactericidal determinations as much as changes in the activity of the normal antimicrobial system. It would be of interest to determine whether changes in the concentration of Mn^{2+} in blood and other tissues in man and animals have any bearing on unexplained differences in their susceptibility and resistance to *Brucella* infection.

Sufficient Co^{2+} became available from 10 mg of exchange resin in 10 ml of blood to inhibit the growth of large numbers of *Br. abortus* and *Strep. agalactiae*. By employing the method of Burstall et al. (1950), approximately 17 μg of Co^{2+} were found in 1 ml of serum from 10 ml of defibrinated blood previously treated with 10 mg of a Co^{2+} resin. The theoretical amount of Co^{2+} available from 10 mg of Dowex 50-XI is 1334 μg.

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RÉSUMÉ

On a estimé pendant longtemps, en effectuant les épreuves de dépistage et de diagnostic de certaines infections, telle la brucellose, que l'absence de développement des micro-organismes pathogènes dans le sang additionné d'un milieu de culture adéquat, indiquait l'absence d'infection. Cette conclusion était erronée. En effet, la croissance microbienne ne se produit que si le milieu nutritif ajouté au sang contient des substances neutralisant un des facteurs du système antimicrobien du sang, en particulier des ions hydrogène, fournis par les résines échangeuses d'ions.

Dans une précédente étude parue dans le Bulletin, l'auteur avait publié les résultats de ses recherches sur la culture de *Brucella abortus*, dans les milieux au sang. Il a poursuivi ses investigations avec d'autres micro-organismes, Gram positifs et Gram négatifs, et analysé le rôle des résines.

Dans le présent article, l'auteur décrit une méthode simple permettant d'assurer la croissance rapide de micro-organismes dans le sang, sans addition d'un milieu de culture liquide. Elle consiste à ajouter au sang des quantités optimum de résines échangeuses d'ions hydrogène et magnésium, ainsi que du citrate de sodium. Les bactéries exigeant la présence de CO_2, se multiplient rapidement aussi, si les flacons de culture sont fermés durant l'incubation. Un supplément de CO_2 est fourni par la décomposition du bicarbonate de sodium du sang, à pH 6.

Une légère augmentation de la teneur du sang en ions manganèse stimule nettement la croissance des Brucella *in vitro*. L'auteur suggère que la présence de ce cation dans le sang, en quantité supérieure à la normale, pourrait être un des facteurs de la moindre résistance de certains sujets humains ou animaux à l'infection brucellique.

En revanche, l'augmentation de la teneur en ion cobalt confère au sang un pouvoir bactéricide net à l'égard de nombreuses souches de Brucella et de *Streptococcus agalactiae*.

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